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Project Summary

Tracheal Organ Culture as Air Pollution Damage Indicator

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This report presents the results of a study conducted to determine the effects of various energy-related effluents on respiratory tract epithelial tissue. Measurement of mucociliary activity and characterization of the morphological alterations induced by such effluents was carried out in hamster tracheal organ culture. Different combinations of *in vivo* and *in vitro* exposure and/or maintenance were used to determine the relationship between *in vivo* exposure danger and adverse effects observed in organ tissue.

The pollutants assayed included particulate effluents from mobile and stationary sources of both conventional and advanced energy processes. Included were fly ash (from coal-fired and oil-fired sources), cigarette smoke condensate, and diesel fuel exhaust extract, with benzo(a)pyrene serving as a positive control.

Both acute (72-hr) and long-term (14-day) studies permitted assessment of tissue specific effects. In addition to acute and long-term toxicity studies, testing was conducted to determine the effects of selected particulate effluents on the pathogenesis of viral infections.

This Project Summary was developed by EPA's Health Effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

The Health Effects Research Laboratory of the U.S. Environmental Protection Agency is presently involved in research to evaluate the health effects of human exposure to airborne environmental pollutants. Past research has established a link between exposure to air pollution and an increased frequency and severity of acute respiratory disease. Under certain exposure conditions, environmental pollutants may impair the function of important defense mechanisms of the respiratory tract epithelial tissue. Pathologic consequences could include shifts in normal cell populations, structural abnormalities of cells and cell organelles, and changes in normal cell repair processes. These adverse effects could render the tracheobronchial epithelium more susceptible to respiratory infections, as well as more vulnerable to non-infectious pollutant agents.

Many of the most dangerous particulate emissions commonly found in the ambient air are produced by conventional and advanced energy processes, such as the combustion of coal and oil. Thus, examination of the health effects resulting from fugitive emissions from these processes is becoming increasingly important.

Summary

In support of EPA's research program in this area, IIT Research Institute conducted a study to obtain data on hazardous energy-related pollutants suspected to interfere with normal respiratory function. Although examination of the

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health effects of potential environmental pollutants has traditionally been based on chronic rodent bioassays, these bioassays are complex and require control of many variables over several years' time. Thus, IIT proposed to utilize *in vitro* methods in hamster tracheal organ culture to provide a more rapid, sensitive, and relevant test system for screening a variety of hazardous pollutants. In order to test the suitability of the *in vitro* method as a rapid screening model, both *in vivo* and *in vitro* studies of pollutant exposure effects on tracheal mucociliary transport parameters and cytopathology were conducted and the results compared.

The primary pollutant used in the study was coal-fired fly ash, obtained from a coal-fueled power plant using Eastern Kentucky coal. Additional particulate pollutants tested in tracheal organ culture were oil-fired fly ash, diesel fuel exhaust extract, and cigarette smoke condensate. These air pollutants contain a variety of chemical carcinogens, the most commonly recognized of which is the well known carcinogen benzo(a)pyrene. In order to establish the relative potency of the various test substances, the effect of benzo(a)pyrene on tracheal organ culture was compared to the effects observed for the other pollutants. Thus, benzo(a)pyrene served as a positive control for the study.

Cytotoxicity resulting from exposure to test compounds was expressed as alterations in ciliary activity and cell morphology. Both the short-term and long-term toxic effects of the pollutants on these factors were investigated. The primary indicator of pollution damage used was cilia beating frequency. Morphological changes were detected using light microscopy and scanning electron microscopy.

Suckling and adult golden Syrian hamsters were employed in the *in vivo* studies. In addition, whole tracheal explants from donor Fischer 344 rats and Alexander B10 F_1D hamsters were inserted with fly ash pellets and transplanted through subcutaneous grafting to recipient rats and hamsters. For *in vitro* studies, tracheal ring explants and whole tracheal explants were prepared and maintained in culture. Tracheal organ culture techniques were developed to maintain differentiated respiratory tract epithelial tissue.

Cilia beating frequency was measured for each tracheal explant at four separate areas on the lumen side, and the average recorded as beats per minute. The cilia beating frequency of whole tracheas was observed by focusing on the mucosa through the explant. After an initial incubation period, the baseline cilia beating frequency for each explant was determined.

In vivo inhalation exposure to respirable-size coal-fired fly ash was accomplished using suckling and adult hamsters. The hamsters were exposed to fly ash in a 432-liter capacity plastic chamber at a mass concentration of 2 mg/m^3 for 3 h/day, 5 days/week for 2 weeks. The chamber was maintained at 25-29°C and 30-55% RH. Mass concentration of the particle aerosol in the exposure chamber was monitored using an aerosol, smoke, and dust photometer. In addition, periodic determination of the aerosol concentration was also made using a condensation nuclei counter. The desired mass concentration for the trials was 2000 μg/m^3 of the fly ash particles; the actual concentration was 1987 μg/m^3.

In vivo exposure of hamsters to coal-fired fly ash resulted in a reduction of cilia beating frequency and cytological alterations in the tracheal mucociliary epithelium compared to ambient air controls. The tracheal epithelium of animals experiencing from five to ten 3-h exposures showed large areas of based cell hyperplasia and stratification. The recovery time from damage to the ciliated epithelium was directly proportional to the number of 3-h exposures.

Whole tracheas from adult B10 F_1D Alexander hamsters were grafted subcutaneously (heterotopic tracheal transplants). Epithelial changes after 2 months in untreated tracheas and tracheas with beeswax (cholesterol [1.9] pellets only) were essentially normal, resembling that of the host's own trachea. Pellets containing coal-fired fly ash induced mild focal hyperplasia and occasional areas of squamous metaplasia. In addition, presarcomatous lesions of connective and cartilagenous tissue was observed.

For *in vitro* inhalation exposure studies, fly ash (coal-fired and oil-fired) was suspended in medium at 5, 10, 50, 100, and 500 μg/ml. Tracheal ring explants were exposed to the fly ash for 1 or 3 h/day, 5 days/week for 2 weeks. Culture dishes were placed in a controlled atmosphere chamber and maintained in 5% CO_2 and balanced air. The chamber was then placed on a platform which rocked at 10 cycles/min, causing the medium containing the fly ash agents to flow continuously over the epithelial surface.

At various intervals, alterations in ciliary activity and cytology were documented by light microscopy and scanning electron microscopy. Tracheal cultures exposed for 1 or 3 h/day showed a decrease in ciliary activity that paralleled cytopathological changes following seven exposures to 50 μg oil-fired fly ash/ml or higher. Morphological alterations consisted of diffuse basal cell hyperplasia and stratified metaplastic epithelium. Whole suckling hamster tracheas in organ culture exposed for 1 or 3 h/day to 10 and 50 μg coal-fired fly ash/ml produced cornifying epidermoid metaplasia after seven exposures. The most characteristic finding of surface cells was a number of broad metaplastic areas with keratin for formation.

Oil-fired fly ash produced a more pronounced effect than coal-fired fly ash. Complete cessation of ciliary activity occurred after seven exposures to 50 μg oil-fired fly ash/ml. The epithelium of cultures exposed to 10 μg/ml or greater of oil-fired fly ash showed pathological alterations proportional to the concentration of fly ash and the period of time exposed. Histological sections of treated explants showed distinct cytopathologic changes which preceded ciliostasis.

In vitro exposures of hamster tracheal epithelium to cigarette smoke condensate (CSC) and diesel fuel exhaust extract (DFEE) were performed to elucidate functional and morphological changes. Early expression of the cytotoxicity of both materials was indicated by altered ciliary activity and cytopathology. Controls exposed to DMSO (DFEE solvent) and acetone (CSC solvent) showed changes in the mucosa at a concentration of 0.25% and 0.2% nine days after initial exposure. At 50 and 100 μg cigarette smoke condensate or diesel fuel exhaust extract, histopathological alterations included stratified metaplastic epithelium (33%) and epidermoid metaplasia (20-25%). At these concentrations, ciliary activity and cytopathology were not altered. Ring explants exposed to 1 and 3 μg benzo(a)pyrene/ml, the prototype carcinogenic polycyclic aromatic hydrocarbon and a positive control for the organ culture bioassay, showed epithelial changes progressing to stratified metaplasia. These changes were reflected as an alteration in cytopathology and a decrease in beating frequency.

In separate experiments, *in vivo* and *in vitro* tests were used to determine the interactions of fly ash and influenza virus (e.g., what effect the virus had on the expression of fly ash damage).

In vivo experiments to study the interaction of fly ash and influenza A/PR-8 virus were conducted using adult male golden Syrian hamsters. In the first experiment, influenza virus replication was measured in tracheal epithelium after aerosol infection. Virus aerosol infection of hamsters was immediately followed by exposure to fly ash in the second experiment. The third experiment was conducted similarly to the second, except that fly ash exposure was delayed until one day after virus infection. In the last experiment, hamsters were exposed to fly ash prior to infection by the virus.

Results from the four *in vivo* experiments indicated that hamsters exposed to a pollutant during an on-going infection (i.e., infected by aerosolized influenza virus and 24 h later exposed to 1 mg fly ash/m^3 for 3 h/day, 5 days/week for 2 weeks) produced greater cytotoxic effects than hamsters exposed to fly ash 1 or 2 weeks prior to virus infection. Toxicity was mainly expressed as cytopathological and histopathological alterations along the luminal surface.

Tracheal cultures from hamsters infected with influenza virus aerosol and then exposed *in vitro* to 10 or 50 ug coal-fired fly ash/ml showed morphological alterations that were similar to those observed in the *in vivo* experiments. In comparison to *in vitro* exposure to fly ash alone, histological changes in the virus-fly ash treatment group were less severe. Initial changes were characterized by focal loss of cilia and sloughing of superficial epithelium. Moderate epithelial vacuolation was observed. Only occasional areas of stratified metaplastic epithelium appeared, indicating the virus retards metaplastic changes. These changes were the same whether exposed immediately after infection or 24 h post-infection. Maximum infectious virus titers in fly ash treatment groups were reached earlier than in the virus infected control explants.

Conclusions

The results of this study indicate that cilia beating frequency and cytopathology alone are not adequate as pollution damage indicators. To detect the full extent of cytological damage, these indicators should be used in conjunction with light microscopy and scanning electron microscopy examinations.

Toxicity testing results showed that hamster tracheal organ cultures can be used to study acute effects of fly ash on respiratory ciliated epithelium, and that the toxic effects produced by the two fly ash species are the result of different mechanisms of inducing injury. The toxicity of oil-fired fly ash was found directly proportional to the concentration and duration of exposure. Morphological observations indicated that cytotoxic concentrations of oil-fired fly ash were below the level that produced ciliostasis. In addition, the transformation of tracheobronchial epithelium to epidermoid metaplasia by coal-fired fly ash was shown to be similar in response to the effects of carcinogens, vitamin A deficiency, and nutritional influences *in vitro*. Results also indicated a correlation between fly ash-induced changes in tracheobronchial epithelium of hamsters exposed *in vivo* (2 mg/m^3) and effects observed in hamster tracheal epithelium exposed in organ culture (5-10 μg/ml).

The organ culture assay permitted toxicity ranking of the pollutants tested. In order of decreasing toxicity, they were: oil-fired fly ash, cigarette smoke condensate, coal-fired fly ash, and diesel fuel exhaust extract.

Studies of the effect of influenza A/PR-8 virus on the expression of fly ash damage demonstrated that tracheal explants infected 24 h prior to coal-fired fly ash exposure produce a significantly greater response than explants exposed immediately after infection. These findings may have bearing on the hypothesis that influenza may stimulate the transformation of tracheobronchial epithelium to epidermoid metaplasia by (1) altering the permeability of target cells, so that the penetration of the fly ash is facilitated, (2) increasing the mitotic activity of the cells providing a favorable condition for an attack by the aromatic hydrocarbons and trace elements that constitute the soluble fraction of the fly ash, or (3) interfering with the detoxification mechanisms of the target cells, leading to an increased persistance of the aromatic hydrocarbons.

Environmental exposure to airborne particulates has been linked to chronic respiratory disease, such as chronic bronchitis. Upon inhalation, pollutants are deposited to a large extent in the trachea where they are removed by mucociliary mechanisms, or phagocytized. The effects of the various pollutants on cells of the tracheobronchial epithelium were in many instances complex. Each of the real-world pollutants assayed produced a unique response, probably related to chemical composition and size of the particles. It is recognized that smaller particles are phagocytized by mucosal cells while the larger particles provoke hyperplastic and metaplastic changes. One might speculate that exposure to the particulate pollutants affects the structure and functional continuity of the epithelium, thereby contributing to chronic bronchitis.

Recommendations

Organ cultures of hamster trachea consisting in part of mucociliary epithelium have permitted study of the effects of toxic agents on this tissue. The ring and whole tracheal organ culture preparations were found to be well suited to evaluate a number of parameters on viable explants, as well as fixed tissue. These cultures were maintained for weeks and monitored while being exposed for a specific period under completely defined conditions. The ability to prepare a large number of tracheal cultures permitted multiple replicate exposures at each experimental point and a wide range of concentrations. Our results demonstrated that changes in any single parameter were insufficient indications of the toxic effects of exposure to a test substance, and we therefore recommend that cilia beating frequency data be correlated with morphological and biochemical parameters.

The increasing number of pollutants being emitted into the environment and the backlog of potentially hazardous chemicals used in industry make a rapid, reproducible, and relatively inexpensive test system for evaluating toxicity a pressing need. Hamster tracheal organ cultures provide the necessary criteria to facilitate identification of these toxic agents. Additional investigations are therefore needed to determine the response of mammalian respiratory tract tissue to a variety of complex mixtures.

The studies should be expanded using organ cultures of tracheal epithelium and heterotopic tracheal grafts as a bioassay for respiratory tract carcinogenicity to evaluate environmental pollutants as potential carcinogens. The criteria for assessment would include histologic and cytologic changes, DNA synthesis, and tumor production. In this

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way, the questions concerning the interrelationships between differentiation, cell population kinetics, and various stages in carcinogenesis could possibly be clarified.

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The complete report, entitled "Trachael Organ Culture as Air Pollution Damage Indicator," (Order No. PB 81-168 999; Cost: $9.50, subject to change) will be available only from:

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